Technical Advance

DNA Degradation Test Predicts Success in Whole-Genome Amplification from Diverse Clinical Samples

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The need to apply modern technologies to analyze DNA from diverse clinical samples often stumbles on suboptimal sample quality. We developed a simple approach to assess DNA fragmentation in minute clinical samples of widely different origin and the likelihood of success of degradation-tolerant whole genome amplification (restriction and circularization-aided rolling circle amplification, RCA-RCA) and subsequent polymerase chain reaction (PCR). A multiplex PCR amplification of four glyceraldehyde-3-phosphate dehydrogenase amplicons of varying sizes was performed using genomic DNA from clinical samples, followed by size discrimination on agarose gel or fluorescent denaturing high-performance liquid chromatography (dHPLC). RCA-RCA followed by real-time PCR was also performed, for correlation. Even minimal quantities of longer PCR fragments (~300 to 400 bp), visible via high-sensitivity fluorescent dHPLC or agarose gel, were essential for the success of RCA-RCA and subsequent PCR-based assays. dHPLC gave a more accurate correlation between DNA fragmentation and sample quality than agarose gel electrophoresis. Multiplex-PCR-dHPLC predicted correctly the likelihood of assay success in formalin-fixed, paraffin-embedded samples fixed under controlled conditions and of different ages, in laser capture microdissection samples, in tissue print micropeels, and plasma-circulating DNA. Estimates of the percent in-

formation retained relative to snap-frozen DNA are derived for real-time PCR analysis. The assay is rapid and convenient and can be used widely to characterize DNA from any clinical sample of unknown quality. (*J Mol Diagn* 2007, 9:441–451; DOI: 10.2353/jmoldx.2007.070004)

Archival specimens represent a vast resource for discovery and evaluation of prognostic DNA markers. In the United States alone, there are more than 300 million archived tissue samples with ~20 million samples added annually. These archived samples contain a wealth of genetic information and offer a great potential for discovery and analysis of biomarkers with diagnostic and therapeutic significance.^{1,2} Laser capture microdissected (LCM) samples,³ tissue print micropeels,⁴ and plasma-circulating DNA⁵ are additional sources of valuable clinical material with major potential for discovery and evaluation of cancer biomarkers.

However, the quantity and condition of the DNA trapped in such diverse clinical samples has proven to be a significant barrier.¹ Knowledge of DNA quality is important to determine the types of techniques that the material can support. For example, the quality of archived specimens is dependent on fixation and storage conditions and can be highly variable between samples.^{1,6} In addition, because specimen yield is often a limiting factor in studying nonrenewable clinical samples, the ability to assess DNA quality with a minimal amount of material before investing time and resources for sample analysis

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is of paramount importance.⁷ Recent advances in whole genome amplification (WGA)^{8–15} have made it possible to gain access to sufficient quantities of DNA from clinical samples to perform a number of downstream applications including real-time quantitative polymerase chain reaction (QRT-PCR), comparative genomic hybridization (CGH), and single nucleotide polymorphism (SNP) microarrays.^{8–14} The success of WGA itself also depends on sample quality.^{9,16} Certain types of WGA such as multiple displacement amplification perform well with fresh or snap-frozen (intact) DNA,^{17–19} but their yield⁹ and accuracy^{20,21} diminish on DNA fragmentation. WGA methods that can tolerate various degrees of sample degradation have been recently developed.^{9,14,16} For example, restriction and circularization-aided rolling circle amplification (RCA-RCA), which circularizes fragmented DNA and then applies rolling-circle-initiated multiple displacement amplification, can produce WGA of partially degraded DNA from archived genomes sufficient in both fidelity and yield for quantitative PCR and copy number analysis via microarray-based comparative genomic hybridization.^{9,22} Similarly, plasma-circulating DNA can be amplified via RCA-RCA and used in expanded genetic screening.²¹ However, the investment required for performing such procedures is not justified if there is no guarantee on the suitability of the starting DNA. A simple assay that predicts success of WGA as well as downstream assays for small amounts of starting DNA such as that obtained via LCM, tissue print micropeels,⁴ or plasma-circulating DNA would be very useful because it would prevent wasting time, effort, and resources.

Ethidium bromide-based agarose gel electrophoresis requires more than 100 ng of DNA to visualize the extended fragmentation associated with archived DNA and does not consistently predict success of PCR from archived samples.²³ Methods that use competitive PCR amplification of short-versus-long amplicons have been used for assessing DNA quality and fragmentation.^{7,23–26} However, most cannot address the requirements posed by the minute starting material obtained via microdissection of tumor samples. Published multiplex PCR predictor assays require 100 ng of starting material,²³ but DNA amounts extracted from LCM samples are usually less than 10 to 20 ng.^{3,20} Rapid amplification of polymorphic DNA (RAPD-PCR)-based assays⁷ use a few nanograms of starting material but examine genomic regions outside housekeeping genes. Because tumor samples are frequently altered because of genomic instability,²⁷ RAPD-PCR-amplified sequences cannot distinguish between deletion of chromosomal regions and changes in sample quality in cancer samples.

We describe a housekeeping gene-based multiplex-PCR approach that, when combined with fluorescence detector-based denaturing high-performance liquid chromatography (dHPLC), possesses the sensitivity required to assess sample quality from 1 ng of genomic DNA after a single multiplex PCR reaction. Alternatively, the method may also be used with a common ethidium bromide-stained agarose gel when accurate quantification is not a major issue. Starting with 1 ng of genomic

DNA, a multiplex PCR is performed, and the PCR product is screened via dHPLC or an agarose gel (Figure 1). The use of high-sensitivity fluorescence detection enables easier identification of small amounts of intact DNA exceeding 300 to 400 bases that is instrumental for the success of WGA via RCA-RCA and downstream assays such as quantitative PCR analysis (Figure 1). We report the establishment and validation of this new dHPLC-based approach for accurately assessing DNA quality in archived samples of varying fixation conditions, age, and degradation as well as in minute amounts of LCM, tissue micropeels, and plasma-circulating DNA.

Materials and Methods

Sources and Extraction of Human Genomic DNA

Reference human male genomic DNA was purchased from Promega (Madison, WI). High-grade glioblastoma/glioma and colon cancer formalin-fixed, paraffin-embedded (FFPE) specimens (times after fixation/FFPE age: ~5 to 7 years and ~10 to 12 years, respectively) were obtained from Medical Oncology, Dana Farber Cancer Institute and Brigham and Women's Hospital. Plasma samples from radiation therapy cancer patients were obtained from Radiation Oncology, Brigham and Women's–Dana Farber Cancer Center. The collection and use of unidentifiable human specimens for genetic analysis was approved by the institutional review board. Genomic DNA from snap-frozen tissues was extracted and purified with the DNAeasy kit (Qiagen, Valencia, CA). Genomic DNA was prepared from FFPE glioma and colon specimens using a modified method. In brief, ~25 mg of tissue per sample was deparaffinized by treatment with mixed xylenes (1.2 ml; vortexed, centrifuged 3 minutes at room temperature, removal of xylene, and repeated one to two times until clear), and xylenes were removed by addition of 100% ethanol (1.2 ml; vortexed, centrifuged 3 minutes at room temperature, removal of ethanol, and repeated one to two times until clear). After vaporization of ethanol for 10 minutes at 37°C, samples were washed in phosphate-buffered saline (PBS) (1.2 ml; vortexed, centrifuged 3 minutes at room temperature, removal of PBS). Tissue was placed in 360 μ l of lysis buffer (Qiagen) and 40 μ l of proteinase K (Qiagen) and rotated at 55°C for 24 to 72 hours as needed for full digestion. Subsequent DNA purification was performed using the DNAeasy kit, adjusting buffer and extraction volumes for the volume of lysis buffer used. DNA extracted from FFPE specimens was initially evaluated by gel electrophoresis of 0.75 μ g of DNA in a 1% agarose gel. To extract plasma-circulating DNA, whole blood was centrifuged at 2000 $\times$ g for 15 to 30 minutes within 2 hours of collection, and plasma was carefully collected from the top of the supernatant, as we described earlier.²¹ Plasma-circulating DNA was purified from plasma with QIAamp MinElute virus spin kit (Qiagen) and quantified using the PicoGreen method (Molecular Probes, Eugene, OR) and via real-time PCR for the GAPDH gene as was described previously.²¹

Figure 1. Procedure to assess DNA quality via a multiplex PCR step followed by dHPLC or agarose gel analysis, using ~1 ng of genomic DNA from diverse clinical samples. Samples of optimal quality are amplified via rolling circle-based WGA and processed for downstream assays. Samples of suboptimal quality are not processed further.

To obtain DNA from snap-frozen tumor tissue and from the corresponding FFPE specimens, snap-frozen human breast cancer tissue was dissected into equal-sized fragments. Some fragments were retained frozen, whereas others were fixed in neutral buffered formalin (10%) for 24 hours. After processing, all fragments were sectioned at 50- μ m thickness and processed for DNA extraction.

To obtain DNA from tumor tissue samples kept at room temperature for varying waiting times before fixation and for varying times within formalin, we used xenograph tumors from the human MCAS ovarian cell line grown *in vivo*. MCAS cells (0.5×10^6) mixed with Matrigel (BD Biosciences, San Diego, CA) were injected subcutaneously in the right flank of an 8-week-old female nude mouse (nu/nu; Charles River Laboratories, Wilmington, MA). After 4 weeks the subcutaneous tumor was harvested and dissected into 10 equally sized fragments. One fragment was immediately snap-frozen and retained at -80°C . Three tumor fragments were fixed immediately in 10% neutral buffered formalin for 2, 24, and 48 hours, respectively, before embedding in paraffin. A second and third group of tumor fragments were retained at room temperature for 1 hour and 5 hours, respectively, in PBS before fixation and paraffin embedding in an identical manner. After processing each block was sectioned at a thickness of 50 μm .

For LCM samples, snap-frozen prostate tissues were cut on membrane slides (Molecular Devices Corp., formerly Arcturus Bioscience, Sunnyvale, CA) at 7- μm thickness. The slides were stained with HistoGene LCM frozen section staining kit (Molecular Devices) and then processed via LCM (Veritas Instrument; Arcturus Bioscience Inc.). Alternatively, manual microdissection was performed on the same tissue sections. The captured samples were treated for DNA extraction via overnight proteinase K digestion at 56°C followed by extraction with phenol/chloroform and precipitation with 0.5 mol/L ammonium acetate, glycogen, and 2 vol of 100% ethanol. The DNA was then eluted in Tris 10 mmol/L, pH 8.0, and quantified by Quant-iT PicoGreen double-stranded DNA reagent and kits (Molecular Probes-Invitrogen).

Prostate cancer tissue print micropeels were obtained from fresh tissue slices obtained from radical prostatectomy specimens at the time of surgery, as we reported previously.⁴ Prostate tissue prints were snap-frozen immediately on collection and stored at -80°C before processing; each tissue print was sliced into a set of 5×5 -mm tiles for biomarker extraction. Print collection and processing was performed in an oriented manner (with fiduciary markers) so that FFPE samples from corresponding tumor sites could be identified for DNA analysis. Biological material was extracted from the tissue print

nitrocellulose by immersing the frozen tissue print tile in a buffer containing guanidine-isothiocyanate (Qiagen buffer RLT) and applying mechanical agitation. After removal of insoluble debris, separate DNA and RNA fractions were purified from the tissue print extract using the Qiagen AllPrep DNA/RNA kit. Each 5 × 5-mm prostate tissue print tile produced ~1 to 2 µg of DNA. The corresponding FFPE prostate tumor samples were less than 6 months old at the time they were extracted for DNA following the procedure described above.

Multiplex PCR for Assessing Sample Quality on Agarose Gel and dHPLC

Multiplex PCR to assess sample quality was performed using ~1 ng of purified DNA from fresh or archived DNA, or alternatively using 1 µl of plasma-circulating DNA. The forward and reverse primers used to co-amplify genomic segments of 105, 236, 299, and 411 bp, respectively, from the *GAPDH* gene (GenBank ID J04038) were as follows: 5'-GGCTGAGAACGGGAAGCTTG-3' and 5'-ATCCTAGTTCCTCCCCAAA-3' (105 bp); 5'-CGGGTCTTTGCAGTCGTATG-3' and 5'-GCGAAAGGAAAGAAAGCGTC-3' (236 bp); 5'-AGGTGAGACATTCTTGCTGG-3' and 5'-TCCACTAACAGTCAGCGTC-3' (299 bp); and 5'-TGAATGGGCAGCCGTTAGGAAAGC-3' and 5'-AGACACCCAATCCTCCCGGTGACA-3' (411 bp). A 25-µl PCR reaction was set up that included a final concentration of 0.24, 0.60, 1.2, and 0.30 µmol/L of the four primers 105, 236, 299, and 411 bp, respectively, 300 µmol/L dNTP, 1.5 mmol/L MgCl₂, 1× PCR buffer, and 0.25 µl of GoTaq polymerase 9 (Promega) and the interrogated genomic DNA. Alternatively, JumpStart Taq polymerase from Sigma, St. Louis, MO, was used for some experiments. Thermocycling was performed in a MiniOpticon machine (Bio-Rad, Hercules, CA) using the following program: 94°C for 2 minutes, followed with 40 cycles at 94°C for 30 seconds then 56°C for 30 seconds and 72°C for 1 minute. Then a final extension at 72°C for 3 minutes was performed. PCR products were analyzed either by ethidium bromide-stained 1% agarose gel electrophoresis or via HPLC chromatography on a WAVE dHPLC system (Transgenomics, Inc., Omaha, NE). The WAVE system is equipped with a fluorescence detector and with two 96-well autosamplers that enable high-throughput analysis of PCR products. The use of the high-sensitivity fluorescence detector in conjunction with SYBR Green I dye infusion enables detection and quantification of picogram amounts of PCR amplicons. The dHPLC was run at nondenaturing temperatures (50°C) for analysis of multiplex PCR products.

RCA-RCA WGA

Restriction and circularization-aided rolling circle amplification (RCA-RCA) was used for WGA of genomic DNA extracted from snap-frozen and archived clinical samples. The published RCA-RCA protocol⁹ was used with minor modifications. In brief, 20 to 50 ng of genomic DNA was digested with 5 U of *NlaIII* restriction endonuclease (0.5 µl from a 10 U/µl stock, catalog no. R0125S; New

England Biolabs, Beverly, MA) in 10 µl of 1× T4 DNA ligase buffer (catalog no. M0202M; New England Biolabs) for 2 hours at 37°C. The enzyme was then inactivated via heating at 65°C for 20 minutes. The digested DNA was circularized by adding 0.5 µl of T4 DNA ligase (200 U), 0.5 µl of T4 ligase buffer, and 4 µl of water to the digested DNA solution and incubating at room temperature for 2 hours. T4 DNA ligase was then inactivated by heating at 65°C for 10 minutes. Linear DNA was then eliminated with 1.2 µl of *Lambda* exonuclease and 0.3 µl of *Exonuclease I* (New England Biolabs) in a volume of 25 µl at 37°C for 1 hour. The balance of the volume to 25 µl was made up in *Lambda* exonuclease buffer. The circularized ligation product was then used in a standard multiple displacement WGA reaction using the GenomiPhi kit reagents (catalog no. 25-6600-01; Amersham Biosciences, Buckinghamshire, UK). Specifically, 1 to 2 µl of circularized DNA was mixed with 9 µl of GenomiPhi buffer, heated at 95°C for 3 minutes, and then chilled on ice. An additional 9 µl of GenomiPhi reaction buffer and 1 µl of Phi29 polymerase enzyme mix was then added and incubated at 30°C overnight. The sample was then digested with *NlaIII* enzyme before performing further assays.

For WGA of plasma-circulating DNA, the RCA-RCA protocol was modified as recently reported,²¹ to replace *NlaIII* digestion with T4 DNA polymerase treatment that generates blunt DNA ends. Briefly, 2 to 4.5 µl of plasma-circulating DNA (~2 to 5 ng total) was blunted with 0.3 U of T4 DNA polymerase (New England Biolabs) at 12°C for 15 minutes in 5 µl of 1× T4 DNA ligase buffer (New England Biolabs), supplemented with dNTP (Applied Biosystems, Foster City, CA) at a final concentration 100 µmol/L. The T4 DNA polymerase was then heat-inactivated at 75°C for 20 minutes. The blunted DNA was ligated with 0.25 µl of T4 DNA ligase (2000 U/µl; New England Biolabs) in a volume of 5.25 µl at room temperature for 2 hours. Ligase was heat-inactivated at 65°C for 10 minutes. The sample was then amplified using the GenomiPhi kit for WGA. Nine µl of random hexamer-containing buffer was added to 5.25 µl of ligated DNA and heated at 95°C for 3 minutes to denature the template and then cooled rapidly on ice. The mixture of 9 µl of reaction buffer plus 1 µl of enzyme mix was added to the cooled sample. The reaction mixture was incubated at 30°C for 16 hours. Finally, the reaction was stopped via heating at 65°C for 10 minutes. This modified RCA-RCA protocol can be conducted in a single tube with no intermediate purification steps.

Anti-Primer Quenching-Based Quantitative Real-Time PCR (aQRT-PCR) for 20 Gene Regions and TaqMan Real-Time PCR for 4 Gene Regions

Real-time quantitative PCR using a recently developed anti-primer quenching method²⁸ was used to assess the result of WGA of genomic DNA in 20 gene regions representing different human chromosomes. The aQRT-PCR

Table 1. Primers and Probes Designed for Real-Time PCR (aQRT-PCR) in 20 Gene Regions Covering 14 Chromosomes

Gene	Primer sequence	
<i>Her2</i>	Forward	5'-AGTGCTATCCGAGGGAATGACATGGTTGGGACTCTTGAC-3'
	Reverse	5'-TGACATGGTTGGGACTCTTGAC-3'
<i>PCK1</i>	Forward	5'-CGAGAGAGAGATCCTTGCCCTT-3'
	Reverse	5'-AGTGCTATCCGAGGGAATTCAGATCTGCTCACGGTGT-3'
<i>RAE1</i>	Forward	5'-AGTGCTATCCGAGGGAATATTTCCCTATGTTTGGGGTG-3'
	Reverse	5'-ACAAATTCGAATGGCATAACC-3'
<i>HoxB5</i>	Forward	5'-AGTGCTATCCGAGGGAACCGAGAAGGAGTTTACAAAGT-3'
	Reverse	5'-CGCATACATAGCAAAACGAA-3'
<i>TOP1</i>	Forward	5'-AGTGCTATCCGAGGGAAGACAGCCCCGGATGAGAAC-3'
	Reverse	5'-AAGAATTGCAACAGCTCGATTG-3'
<i>HB-EGF</i>	Forward	5'-AGTGCTATCCGAGGGAACCCAGTTGCCGTCTAGGA-3'
	Reverse	5'-CGGACATACTCTGTTTGGCACTT-3'
<i>E2F1</i>	Forward	5'-AGTGCTATCCGAGGGAATGGCTGGGCGTGTAGGA-3'
	Reverse	5'-CGCTCCATTAAAGCTTCAATCA-3'
<i>TBP</i>	Forward	5'-AGTGCTATCCGAGGGAAGGGCATTATTTGTGCACTGAGA-3'
	Reverse	5'-AGCAGCACGGTATGAGCAACTGTCTAGA-3'
<i>RAN</i>	Forward	5'-AGTGCTATCCGAGGGAATGGAGCCAGCGTCAGA-3'
	Reverse	5'-CGCTGCACCGCTGACAT-3'
<i>TFR</i>	Forward	5'-AGTGCTATCCGAGGGAAGCCATGAGGTCTGAAATGGA-3'
	Reverse	5'-GGCCTTATTCTTCAATCAACA-3'
<i>CYC</i>	Forward	5'-AGTGCTATCCGAGGGAAGCCATGGAGCGCTTTGG-3'
	Reverse	5'-TCCACAGTCAGCAATGGTGATC-3'
<i>BRCA1</i>	Forward	5'-AGTGCTATCCGAGGGAATCTTCTCTGCCACATACCTG-3'
	Reverse	5'-GGGAGGGACATATGGGA-3'
<i>BRCA2</i>	Forward	5'-AGTGCTATCCGAGGGAACCTCCAGTGCGACCAGA-3'
	Reverse	5'-GCCCTCTTTTGGACTAGCAGA-3'
<i>Myc</i>	Forward	5'-AGTGCTATCCGAGGGAATCCCTCTATGCCTCTATCAT-3'
	Reverse	5'-GGAAAGAAGGGTATTATATGGG-3'
<i>HMSH2</i>	Forward	5'-CGTCGATTCCAGATCTTA-3'
	Reverse	5'-AGTGCTATCCGAGGGAACCTGATAGAGTCGGTAACAAT-3'
<i>Ku-70</i>	Forward	5'-AGTGCTATCCGAGGGAACCTTGGCTGTGGTGTCTTAT-3'
	Reverse	5'-TCCAGCTCCTGTAAGACGTAA-3'
<i>CXCR4</i>	Forward	5'-AGTGCTATCCGAGGGAACCGCTACTGGTTGGGTACT-3'
	Reverse	5'-CGCGTCCATCCTTGCTAAAGT-3'
<i>EGFR</i>	Forward	5'-AGTGCTATCCGAGGGAACAGAGGCAGCTCCGAAGA-3'
	Reverse	5'-AAGCTCCGCTCGCCAAAC-3'
<i>c-MYB</i>	Forward	5'-AGTGCTATCCGAGGGAAGTTTCAGCCACGTCTACC-3'
	Reverse	5'-GTCCTCATCATCCTCGTCA-3'
<i>p53</i>	Forward	5'-AGTGCTATCCGAGGGAAGCAGGCTGACGTCGTGAAG-3'
	Reverse	5'-TCGAGTTCGGCTCCTACCAG-3'

protocol was performed using the published protocol without modifications. Fluorescently labeled primer and nonlabeled primers for 20 genes were designed using Oligo 6 software (Molecular Biology Insights, Cascade, CO) and synthesized by Integrated DNA Technologies (Coralville, IA). A single BHQ-2-labeled anti-primer was synthesized from Integrated DNA Technologies and used for all reactions. The sequences of the primers and anti-primer are listed in Table 1. 6-FAM (FAM) was used as a label at the 5' end of the forward or, alternatively, the reverse fluorescent primer. To validate the performance of individual primers, for each gene tested standard curves were obtained using serial dilutions of DNA (0.14 to 145 ng) as described.²⁸ To perform aQRT-PCR reactions, amplification was performed using the AmpliTaq Gold amplification kit (Applied Biosystems, Branchburg, NJ) in a Smart-Cycler real-time thermocycler (Cepheid, Sunnyvale, CA). Genomic DNA in a 1- to 3- μ l volume was added to a final volume of 20 μ l with a final concentration of 1 $\times$ ABI TaqMan master mix (Applied Biosystems), 0.2 μ mol/L each of fluorescently labeled primer and unlabeled primer, and 1 μ mol/L BHQ-2-labeled anti-primer

(synthesized from Integrated DNA Technologies). The thermocycling program was 50°C for 2 minutes one cycle, 95°C for 10 minutes one cycle, and 40 cycles (95°C for 15 seconds, 60°C for 30 seconds, 50°C for 30 seconds, and 50°C for 15 seconds for reading fluorescence). The TaqMan real-time PCR method for the genes *HER2*, *GAPDH*, *HB-EGF*, and *TOP* was performed as we described previously.⁹

Results

Amplification of GAPDH Sequences Using Multiplex PCR

Multiplex PCR using a number of GAPDH primer combinations was first tested using intact, human male, genomic DNA (reference DNA). The PCR products of the selected primer combination was then examined using two different modes of detection, agarose gel electrophoresis using ethidium bromide staining and dHPLC-based fluorescence detection using SYBR Green I dye. Figure 2 depicts the

Figure 2. Multiplex PCR performed on varying amounts of human male genomic DNA (reference DNA). **A:** PCR products examined via ethidium bromide-stained agarose gel electrophoresis. **B:** PCR products examined via dHPLC equipped with a high-sensitivity fluorescent detector.

PCR products obtained using gel electrophoresis (Figure 2A) or dHPLC (Figure 2B) using 10, 5, or 1 ng of reference genomic DNA. The anticipated PCR products (105, 236, 299, and 411 bp) are visible using both ethidium bromide agarose gel-based and SYBR Green I-fluorescent dHPLC-based detection, for starting DNA of 1 to 10 ng. These two independent detection methods are in agreement, although minor differences are observed in the relative quantification of the PCR amplicons. The reproducibility of these results was tested in independent experiments. The inset in the dHPLC-based visualization using 1 ng of starting material enables a closer look at the background fluorescence relative to the peaks (signal-to-noise ratio) obtained using the high-sensitivity fluorescence detection mode of the dHPLC.

Evaluation of RCA-RCA WGA Using Real-Time PCR

Identification of copy number changes, SNP genotyping, or mutation detection in clinical samples is frequently

Figure 3. Evaluation of the real-time PCR threshold difference (amplification efficiency) before and after WGA via RCA-RCA for 20 randomly chosen genes, using reference DNA. The anti-primer quenching method was used for real-time PCR quantification. For selected genes (*HER2*, *GAPDH*, *TOP1*, and *HB-EGF*) the TaqMan real-time PCR method was also used, for comparison (columns with pattern). Dotted line is the average threshold difference observed between all 20 genes.

conducted via real-time PCR-based methods.^{28–30} A criterion for the success of WGA methods is the ability to perform real-time PCR from randomly selected genomic regions.⁸ To evaluate the modified RCA-RCA WGA protocol, a real-time quantitative PCR for 20 randomly selected genes representing different human chromosomes was applied. RCA-RCA was performed on 20 ng of reference DNA. An aQRT-PCR platform that was recently developed by our group²⁸ was used for signal generation. aQRT-PCR uses short amplicons and is suitable for amplification of DNA from clinical specimens of variable quality (fresh, FFPE, and plasma-circulating DNA).²⁸ Because it uses a singly-labeled primer per amplicon combined with a universal quencher, aQRT-PCR enables low-cost, high-throughput screening and was adopted in this investigation. For a subset of four genes, the TaqMan real-time PCR method was also performed for a direct comparison to aQRT-PCR.

To assess the uniformity of amplification efficiency after RCA-RCA of reference DNA, aQRT-PCR on the 20 selected genes was applied using unamplified and RCA-RCA-amplified DNA. To enable a direct comparison of amplification efficiency, 4 μ l containing a total of 3 ng of genomic DNA were diluted to 100 μ l and directly tested via aQRT-PCR using 1 μ l per reaction. A further 4 μ l containing a total of 3 ng of DNA were processed for RCA-RCA amplification, diluted to 100 μ l, and again tested via aQRT-PCR using 1 μ l per reaction. The difference in amplification threshold (ΔC_t) before and after RCA for the 20 genes studied is depicted in Figure 3. The dotted line represents the mean ΔC_t of all 20 genes. The data indicate that the amplification difference among the 20 genomic regions examined does not exceed a PCR threshold of ± 1.5 -fold, indicating uniform WGA via the modified RCA-RCA protocol. The patterned columns on Figure 3 indicate the results obtained for four genes when real-time PCR using the TaqMan approach is performed. Similar to our previous observation,²⁸ the threshold difference ΔC_t obtained with the TaqMan method agrees well with the ΔC_t obtained using aQRT-PCR. In summary, these data indicate that for DNA for which all four prod-

Figure 4. Examination of the effects on DNA sample quality of wait-time period at room temperature after surgery and time of fixation in formalin. Extracted genomic DNA was amplified via WGA (RCA-RCA) and tested for amplification efficiency at 20 gene loci. **A:** Amplification efficiency versus sample preparation conditions applied. **B:** Ethidium bromide gel-based multiplex PCR test of the extracted genomic DNA. **C:** dHPLC-based multiplex PCR test of the extracted genomic DNA.

ucts are clearly evident in the multiplex PCR test (Figure 2), RCA-RCA results in a uniform WGA from which real-time PCR is successfully conducted with comparable efficiency on all 20 genes studied.

Effect of Formalin Fixation Conditions on WGA and Real-Time PCR

The reality of surgical operating rooms does not always allow tissue processing immediately after tissue resection and as a result samples may remain at room temperature for extended periods of time before fixation. In addition, fixation time in formalin can vary between 2 and 48 hours (ie, a weekend). To examine in a systematic manner the effect that the wait time between surgery and formalin fixation has on DNA quality, an ovarian tumor section from our xenograph samples was snap-frozen immediately after excision and stored at -80°C until DNA extraction. Additional identical tissue sections were processed such that a 0- to 5-hour waiting time elapsed before fixation. In addition, a 2- to 48-hour formalin fixation time was applied on individual sections of the excised ovarian tumor before paraffin embedding. This series of FFPE samples was stored at room temperature for 3 to 4 weeks before DNA extraction.

One ng of DNA from each snap-frozen and FFPE specimen was used for evaluation of sample quality via multiplex PCR. In addition, 20 ng of DNA from each sample was amplified via RCA-RCA, after which aQRT-PCR was applied over the same 20 genes used for reference DNA. Figure 4A compares the aQRT-PCR threshold obtained from each formalin fixation condition to the aQRT-PCR threshold of the snap-frozen sample. The data indicate

that the aQRT-PCR amplification efficiency of the FFPE samples is similar to that of the snap-frozen sample for the fixation conditions applied and that all 20 genes are uniformly amplified via RCA-RCA both for the snap-frozen sample and for the various fixation conditions. Figure 4, B and C, depicts the results of the multiplex PCR for each sample using ethidium bromide-stained agarose gel or dHPLC, respectively. The four multiplex-PCR products are evident in all samples, with minor differences occurring from sample to sample. In conclusion, these data indicate that a wait time of up to 5 hours at room temperature and fixation times of up to 48 hours make no significant difference to DNA quality extracted from FFPE samples. The subsequent WGA via RCA-RCA and real-time PCR are efficient in all 20 genes tested.

Application of WGA and Real-Time PCR to Aged FFPE Samples and Correlation to Multiplex PCR-Based Index of Degradation

To examine the effect of the FFPE sample age (ie, time elapsed since FFPE treatment) on WGA via RCA-RCA and downstream real-time PCR, DNA was extracted from sets of FFPE glioma and colon tumor samples obtained under institutional review board approval from Medical Oncology, Dana Farber Cancer Institute, and from the Department of Pathology, Brigham and Women's Hospital. The ages of the glioma and colon tumor FFPE samples were ~ 5 and ~ 10 to 12 years, respectively. One ng from each FFPE specimen was then used for evaluation of sample quality via multiplex PCR. In addition, 20 ng of DNA from each sample was amplified via RCA-RCA, after

Figure 5. Examination of DNA quality in a range of glioblastoma FFPE samples, ~5 years old, using three different approaches: direct observation of extracted DNA on ethidium bromide gel (A), ethidium bromide gel-based examination of multiplex-PCR products (B), and fluorescence detector-based dHPLC screening of multiplex-PCR products (C). The number of sites that amplified successfully via real-time PCR after RCA-RCA WGA from these samples is also depicted in C (aQRT-PCR score).

which the same 20-gene real-time PCR test described in Figures 3 and 4 was applied using aQRT-PCR in parallel for reference DNA and FFPE samples.

Figure 5 depicts the agarose gel profiles of the unamplified glioma FFPE samples (Figure 5A), gel-based multiplex PCR (Figure 5B), and dHPLC-based multiplex PCR (Figure 5C). Most of the DNA samples from the ~5-year-old glioma FFPE specimens are still fairly intact, according to the agarose gel electrophoresis profile, except for 138, 70-5, and 70-8 (Figure 5A). The multiplex PCR results in Figure 5, B and C, indicate the presence of all four anticipated PCR products in each of the samples except for samples 138, 70-5, and 70-8. In those three cases the longer amplicons were weakened or absent relative to the 100-bp amplicon. The number of genes, of 20 tested, that could be successfully amplified from each glioma FFPE sample by real-time PCR after RCA-RCA amplification is depicted in Figure 5C (aQRT-PCR score). Amplification was defined as successful if a threshold difference of at least 10 cycles was observed when performing aQRT-PCR in samples before and after RCA-RCA. Eighteen to 20 genes were successfully amplified from all FFPE samples except for 138, 70-5, and 70-8. The data indicate agreement between the percentage of genes successfully amplified via RCA-RCA and the degradation status of the ~5-year-old glioma samples as predicted by all three detection methods, direct examination on agarose gel, gel-based multiplex PCR, and dHPLC-based multiplex PCR.

Next, colon tumor FFPE samples of ~12 years age that appeared to be significantly more degraded than the

glioma samples on agarose gel electrophoresis (Figure 6A) were examined by the same methodology. Multiplex PCR was performed and the amplicons screened via ethidium bromide gel electrophoresis and high-sensitivity dHPLC (Figure 6, B and C, respectively) and correlated to the results of aQRT-PCR for the same 20 genes after RCA-RCA. In Figure 6C, the samples were ranked according to the number of genes successfully amplified via RCA-RCA, using the 20-gene real-time PCR test (aQRT-PCR score). Individual samples are listed in the order of decreasing score.

Substantial differences could be seen between the expectations from visual inspection of degradation on agarose gel and the ability to perform RCA-RCA and real-time PCR. For example, samples 7 and 1 had similar agarose gel electrophoresis profiles (Figure 6A), but they ranked clearly different in their ability to PCR amplify (aQRT-PCR scores 17 of 20 and 9 of 20, respectively, Figure 6C). Similar deviations were also observed for samples 2, 3, and 6 that seem comparable on agarose gel electrophoresis. Such deviations can be understood in that formalin treatment generates DNA crosslinks that may inhibit PCR but are not evident by direct examination of the agarose gel profile.²³

When multiplex PCR was performed on the FFPE samples and the products screened using ethidium bromide-based agarose gel (Figure 6B), the assay could differentiate between samples 7 and 1 that seem similar in their agarose gel profiles (Figure 6A) and the agreement with the aQRT-PCR score improves. Nevertheless, subtle differences between sample quality for certain samples

Figure 6. Examination of DNA quality in a range of colon FFPE samples, ~10 to 12 years old, using three different approaches: direct observation of extracted DNA on ethidium bromide gel (A), ethidium bromide gel-based examination of multiplex-PCR products (B), and fluorescence detector-based dHPLC screening of multiplex PCR products (C). The number of sites that amplified successfully via real-time PCR after RCA-RCA WGA from these samples is also depicted in C (aQRT-PCR score). The individual samples in C are listed in the order of decreasing aQRT-PCR score.

were still unclear. For example, samples 4, 6, 1, 3, and 10 that rank substantially differently in the aQRT-PCR score seem similar using the gel-based multiplex PCR. Multiplex PCR followed by fluorescent dHPLC detection enabled visualization of the two longer amplicons with higher sensitivity than the ethidium bromide gel (Figure 6C) and correlated better with the aQRT-PCR score

for the samples examined. Therefore, although both gel electrophoresis and dHPLC could be used in the present approach, the dHPLC data indicated a closer agreement between the results of the 20-gene test on the RCA-RCA-amplified FFPE samples and the prediction of the multiplex PCR.

It is concluded that, for FFPE samples that are significantly degraded, presence of longer PCR amplicons was predictive of the ability of RCA-RCA to amplify genomic DNA from FFPE samples and the success of downstream real-time PCR. The presence of longer amplicons even in small proportion relative to the shorter amplicons resulted in successful amplification of most, or all of the 20 genes tested from RCA-RCA-amplified DNA.

Evaluation of the Fragmentation in Plasma-Circulating DNA Using Multiplex-PCR

Plasma-circulating DNA can be a minimally invasive means for identifying and tracing tumor biomarkers such as mutated oncogenes, methylated tumor suppressor genes, loss-of-heterozygosity, and other tumor-specific genetic changes.^{5,31–35} Further, the degree of fragmentation in plasma-circulating DNA has been reported to bear an inverse correlation to tumor load, with normal individuals being less likely to have large, necrotic fragments of circulating DNA in their bloodstream.^{32,36} We recently demonstrated that WGA of plasma-circulating DNA using a modified RCA-RCA protocol enables an expanded and reliable detection of loss-of-heterozygosity in breast cancer patients.²¹ However, occasionally the plasma-circulating genome obtained from some individuals is too degraded to be successfully amplified. A rapid evaluation of the fragmentation status of plasma-circulating DNA can be useful for practical as well as for diagnostic purposes.

To examine the success/failure of WGA of plasma-circulating DNA and the relation to the multiplex-PCR-dHPLC test developed here, four plasma samples from radiation therapy cancer patients were used. Two of these samples amplified successfully via the modified RCA-RCA protocol, as evidenced via real-time PCR before and after RCA-RCA, and the other two failed to amplify. One μ l of plasma-circulating DNA from each sample was used for multiplex PCR and the products examined via dHPLC. Figure 7A depicts the multiplex PCR amplicons obtained using the high-sensitivity fluorescence detector. The data indicate that the longer amplicons were absent for the two samples that failed to amplify via WGA. On the other hand, all four amplicons were present for the two samples that amplified successfully via the modified RCA-RCA protocol. The presence of the longer amplicons in the multiplex-PCR-dHPLC test was predictive of the success of WGA in plasma-circulating DNA. Finally, Figure 7B depicts representative multiplex PCR-dHPLC results from diverse clinical specimens: a pair of snap-frozen and FFPE-treated breast cancer specimens, a pair of tissue print micropeel, and the corresponding FFPE-treated prostate cancer speci-

Figure 7. Examination of DNA quality in a range of clinical samples using fluorescence detector-based dHPLC screening of multiplex PCR products. **A:** Plasma-circulating DNA from four different colon cancer patients. **B:** 1 and 2, Breast cancer snap-frozen and corresponding FFPE; 3 and 4, prostate cancer micropeel (snap-frozen) and corresponding FFPE; and 5 and 6, prostate cancer LCM and manually dissected specimens.

men, and a pair of LCM- and the corresponding manually dissected prostate cancer specimens.

Discussion

DNA from minute clinical samples (LCM, plasma, tissue print micropeels) often requires WGA before screening with real-time PCR or other downstream technologies. Restriction and circularization-aided whole genome amplification (RCA-RCA) enables unbiased representation of DNA extracted from snap-frozen and degraded FFPE samples as evidenced by microarray-based comparative genomic hybridization screening of clinical samples on cDNA arrays.⁹ However, amplified FFPE samples exceeding a certain degree of degradation are less amenable to successful microarray-based comparative genomic hybridization after RCA-RCA.⁹ Similarly, real-time PCR-based assays, such as TaqMan^{29,30} or aQRT-PCR²⁸ used for genotyping or copy number determination from RCA-RCA-amplified samples, are successful in amplifying DNA from a range of clinical specimens but can fail in highly degraded clinical samples.^{9,21}

To avoid the unnecessary cost and effort associated with failures caused by DNA quality, we developed a multiplex PCR assay for genomic amplicons of increasing size on the GAPDH housekeeping gene. We applied RCA-RCA-amplified DNA in a real-time PCR assay using 20 randomly chosen genes representing 14 different human chromosomes. Because sample degradation accumulates with FFPE sample age, the longer PCR products in the GAPDH multiplex PCR gradually become less visible or disappear (Figure 6). Our data demonstrate that even a very small amount of the longer PCR fragments can be decisive for the success of RCA-RCA WGA and the subsequent real-time PCR-based assays. RCA-RCA utilizes digestion via the 4-bp-recognizing enzyme *Nla*III in the first step of the procedure, and the average separation of any two successive *Nla*III sites is ~250 bp. The

presence of even small amounts of 300- to 400-bp fragments in clinical samples, which are more easily identified via multiplex-PCR-dHPLC, are accordingly important for efficient WGA via RCA-RCA and the assays that follow. This conclusion seems to be generally applicable to DNA obtained from many sources, including plasma-circulating DNA, tissue micropeels, and LCM samples (Figure 7). This report is the first to associate a well-defined multiplex-PCR assay with the success of a specific WGA method as judged by extensive postamplification real-time PCR and to reiterate the significance of sensitive detection of the larger DNA fragments. The methodology developed here may also be used to associate DNA quality with the probability of success of additional WGA methods, such as multiple displacement amplification or OmniPlex,^{8,15} and potentially with different downstream assays, such as single nucleotide polymorphism detection microarrays and aCGH. Alternatively, the method can also be applied for predicting the probability of success of screening FFPE specimens via real-time PCR-based assays in the absence of WGA, when the starting DNA quantity is not an issue.^{37,38} Significantly, our data verify previous reports²³ that a visual evaluation of genomic DNA size using an ethidium bromide-stained agarose gel is not a reliable predictor of DNA quality in samples extracted from FFPE specimens and that a DNA amplification test is superior. Direct examination of DNA sizing on agarose gel electrophoresis is not possible for minute DNA samples, and the ability to perform the present multiplex PCR test from ~1 ng of starting material is critical for microdissected samples.

A variety of factors were examined as potentially influencing DNA quality in FFPE samples (wait time at room temperature before formalin fixation, time in formalin, age of FFPE sample). Wait time and time in formalin have little influence on DNA quality as assessed via multiplex-PCR-dHPLC and by the assays applied after RCA-RCA. FFPE age seems to be the single most important parameter influencing DNA quality for the assays reported. Ghazani and colleagues³⁹ also found a lesser influence of the fixation time in formalin for FFPE samples screened via microarray-based comparative genomic hybridization.

In summary, a sensitive multiplex PCR-dHPLC/ethidium bromide gel assay of DNA quality was developed that can be used with ~1 ng of starting material from diverse, minute clinical samples when DNA is a limited resource. RCA-RCA-amplified DNA from such samples can be used for real-time PCR, and potentially for microarray-based assays, and the probability of success can be predicted via the multiplex-PCR dHPLC/gel assay. The assay is rapid and convenient and can be widely used to characterize DNA from any clinical sample including plasma-circulating DNA.

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